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Method for measuring chemical groups attached to biological molecules

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Related applications

This application claims priority of the Swiss patent application No. CH 0653/03 filed on April 10, 2003.

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Technical field of the invention

The invention relates to a method for measuring chemical groups attached to

25 biological molecules, in particular for measuring the presence or absence of phosphate groups attached to biological molecules.

The classes of enzymes which catalyze the attachment (phosphokinase) and/or the removal (phosphatase) of phosphate residues to and from biomolecules is of

30 great biological and pharmacological importance. In order to measure the efficiency of enzyme functionality it is possible, among other means, to utilize the concentration of reactants or products of the reaction, that is for instance, the quantity of phosphorylated biomolecules in the sample volume.

Methods for measuring the presence and/or absence of chemical groups, for in-
stance of phosphate groups, attached to biological molecules are known per se
and include for instance, the use of fluorescence polarization. Such fluorescence
polarization measurements are based on changes in the volume of the molecules

5   subject to measurement. Many of the reactions involving biological processes
based on an increase in volume, such as those involving receptor-ligand bonds,
antibody-antigen bonds, DNA hybridization or DNA protein bonds, as well as
those processes involving a reduction in volume, such as enzymatic degradation
or dissociation reactions, can be measured directly by means of fluorescence po-

10   larization.

Such tests employ the following approach: An unphosphorylated biomolecule is
incubated with an enzyme which binds a phosphate group to this molecule,
namely with a phosphokinase, or a phosphorylated peptide is incubated with an

15   enzyme that removes the phosphate group from this peptide, that is with a
phosphatase. The change in the volume of a biological molecule by adding or
removing a small chemical group, such as for instance a phosphate, sulfate or
oxalate group, is not significant enough for it to be detected directly by means of
fluorescence polarization. As a rule, in order to enhance the volume effect an

20   auxiliary molecule is attached to the biological molecule being analyzed. These
auxiliary molecules, in the form of antibodies, have to be selected in such a way
that they only ever form a bond with the peptides being analyzed when these
peptides truly include a phosphate group. This ensures that there is a significant
difference in terms of their volume between the phosphopeptide / antibody com-

25   plexes being measured by means of fluorescence polarization and the non-
phosphorylated peptides without any attached auxiliary molecules. This commer-
cially available approach was originally developed to provide a non-destructive
marker which facilitated the tracking of enzyme functionality. In a situation in
which the pharmacological efficacy of chemical substances is being tested (i.e. a

30   drug discovery situation), such assay substances are added to the system, with
the standardized fluorescence polarization measurement providing information as
to whether the assay substance produces the desired modulation effect on the
enzyme or not.

Related prior art

All known methods for ascertaining phosphorylation and/or dephosphorylation
are based on the addition of auxiliary molecules, such as the method for instance

5   disclosed in US 6,410,255. In addition to antibodies, other chemical compounds
can also be employed as the auxiliary molecules for detecting the phosphoryla-
tion of a biomolecule, thus being capable of differentiating one state of phos-
phorylation from another. If one were to attempt to measure the concentration
of other reactants or products of a phosphorylation reaction using an alternative

10   method, one would have to employ chemical methods involving more complex
additions than is the case with fluorescence polarization.

Objects and summary of the invention

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In accordance with the object of the present invention, an alternative method is
proposed for detecting a chemical reaction at a biological molecule in a simple
manner, such as for instance phosphorylation or dephosphorylation of a peptide.

20   This object is achieved by means of the characteristics specified in the independ-
ent Claim 1. Preferred embodiments of the method in accordance with the inven-
tion and/or additional inventive elements are derive from the dependent claims.

It has been known for a long time that the fluorescence polarization signal emit-
25   ted by a sample tagged with a fluorescent marker is not altered significantly sim-
ply by adding or removing a phosphate group and that charges can alter the
fluorescence lifetime of fluorescent dyes. However, the fact that it is sufficient to
add a phosphate group to a biomolecule supplemented with a fluorescent marker
in order to generate a workable signal, was a complete surprise.

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Consequently, the present invention is based upon the unexpected and surprising
discovery that by adding or removing a phosphate group to or from a biological
molecule respectively, the fluorescent lifetime of fluorophores attached to these

samples, reflecting the presence or absence of a phosphate group, is altered significantly.

5   **Detailed description of the invention**

The mechanism upon which the present invention is based is not fully understood. However, it is assumed that it involves an interaction between the fluorescent marker and a charged residue, which is attached in the vicinity of the bind-
10   ing site of the fluorescent marker on the same biomolecule and/or is located within the spatial range of the fluorescent marker. Fluorescein was found to be a fluorescent marker, the fluorescence lifetime of which changes significantly in response to the presence or absence of a phosphate group. However, the invention encompasses not only the use of fluorescein; the general assumption is made
15   that every chemical reaction involving a biomolecule, which produces one or more charged chemical residues, can be detected properly by measuring the fluorescence lifetime. The assumption is made that such or similar results can also be used for establishing the presence of other enzyme reactions, if in accordance with the present invention, the fluorescence lifetime of suitable fluorescent
20   markers attached to the corresponding biomolecules is measured.

It is known that enzymes catalyze many different types of chemical reactions. The following table lists the most common reactions observed in living systems and the associated enzyme classes which play an important role in these reac-
25   tions:

Reaction	Impact	Enzyme class	Enzyme name
Oxidation; Reduction	C–O, C–C or C–N bonds	I	oxidoreductases
Intramolecular and intermolecular transfer	Transfer of functional groups	II	transferases
Hydrolysis	Ester, ether and amides	III	hydrolases
Elimination; Addition	C–O, C–C or C–N bonds	IV	lyases
Isomerization	Steric modifications	V	isomerases
Binding	Ester, thiolester and amide bonds	VI	ligases

Using the method in accordance with the invention, enzyme reactions which enrich molecules by means of a catalytic process can be detected and quantified.

Enzyme class I:

5   Oxidoreductases catalyze redox reactions, with oxidation resulting in the loss of electrons and a reduction in the acquisition of electrons. Consequently, it is possible for instance, that the modification of a fluorescence lifetime signal can be observed for a fluorescent marker which is located in the direct vicinity of an active oxidoreductase site, indicating the migration of electrons through the various

10   amino acid side chains within the enzyme or substrate during the catalytic transition. This fluorescent chain has already been detected in Class I enzymes: the reduction of NAD^+ to NADH by means of dehydrogenases has shown that NAD^+ is not fluorescent, in contrast to NADH.

15   Enzyme Class II:
      Transferases are enzymes which catalyze the transfer of a functional group from one substituent to another, which can occur within the same molecule or between different molecules. Typical examples for this class are kinases, which transform ATP to a protein or peptide. Similar effects involving the fluorescence

20   lifetime, such as for the phosphokinase reactions described below, can be expected for thiolases and other transferases.

Enzyme class III:
      Hydrolases are enzymes which catalyze the hydrolysis of carboxylic acid esters,

25   hemiacetal ethers (glycosyl compounds), thioethers, amides (peptide bonds) and acid anhydrides. This group also includes the phosphatases, as they cleave a phosphate group into water. The information for Class I and II also applies with respect to this class.

30   Enzyme class IV:
      Lyases are enzymes which catalyze the elimination or addition of chemical groups. One example is the elimination of a CO group, known as decarboxylation. Due to the fact that the decarboxylases generally give up a pair of electrons in order to catalyze the decarboxylation reaction, as a change to the "electronic

signature" of the substrate can be anticipated during the course of the decar-
boxylation reaction, this class of reactions and/or enzymes should also be able to
be analyzed using lifetime fluorometry.

5   Enzyme class V:
Isomerases catalyze certain intramolecular rearrangements, such as racemiza-
tion, epimerization, cis-trans-conversions and enol-keto-tautomerization. As
massive electron movement is also expected during enol-keto-tautomerization, it
should also be possible to measure a change in the fluorescence lifetime in this
10   case.

Enzyme class VI:
Ligases are enzymes which catalyze a bond between molecules, using the energy
they gain from cleaving the ATP (or a similar nucleoside triphosphate). This class
15   includes, for instance, fatty acid synthetases and DNA polymerases. Similarly, an
assumption can be made with respect to these enzymes that it is highly likely
that a catalytic reaction produced by these enzymes will result in a significant
change to the fluorescence lifetime of a fluorophore located in the vicinity of an
enzyme's active site.
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In accordance with the present invention a single fluorescent marker suffices if it
is located in the vicinity and/or in the sphere of influence of a chemically active
group on the biomolecule. One condition with respect to the fluorescent marker
is that it reacts to an alteration in the charge density and/or the configuration in
25   its direct molecular environment by altering its fluorescence lifetime. The signal
generated in this manner is thus much more direct than the method representing
the state of the art, which employs the addition and binding of auxiliary mole-
cules.

30   The list of biological molecules (of natural and synthetic origin) provided here in
relation to the present invention is by no means exhaustive and is purely for ex-
emplary purposes:

- molecules generated by biological or synthetic means encompassing an amino acid sequence, such as proteins, peptides, glycoproteins and lipoproteins;

5 - molecules generated by biological or synthetic means encompassing a nucleic acid sequence such as DNA and RNA fragments or oligonucleotides;

- other molecules resulting from a biological process or serving such a process such as cyclical adenosine monophosphate (AMP) or guanosine mono-
10   phoshate (GMP);

- monosaccharides, polysaccharides and other macromolecules.

A multiwell plate is defined in connection with the present invention as an ar-
15 rangement of open or closed chambers. This arrangement is preferably regular and constitutes a grid-like array of sample containers or sample holders. Known multiwell plates of this type are for instance the so-called microplates, with 96, 384 or 1536 wells arranged in a rectangular grid. The samples do not necessarily have to be arranged in depressions. Smaller samples can be also be arranged on
20 a flat surface in an array and be separated from each other simply by hydrophobic sections or small elevations. Preferably, all multiwell plates have in common that a large number of samples with addressable positions can be processed simultaneously or essentially simultaneously.

25 Among others, known methods for measuring the fluorescence lifetime are the phase modulation technique and time correlated single photon counting (TCSPC). The present invention shall now be presented in more detail with reference to a selected exemplary experiment.

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Experiment 1

In this experiment, the known method of time correlated single photon counting (TCSPC) for measuring the fluorescence lifetime was employed.

A)   Technical details:

The experiments were conducted with commercially available chemicals. In doing so, the following chemicals were used:

5   PTK Green as the tracer peptide, concentration 2 nM, P-2843 from PANVERA (PANVERA, 501 Charmany Drive, Madison WI USA), as part of the "green" kinase assay, in accordance with Kit No. P-2837;
PTP1B phosphatase, SE-332 from BIOMOL (BIOMOL Research Laboratories, Inc., 5120 Butler Pike, Plymouth Meeting PA USA 19462-1202);

10   fluorescein and PBS buffer.

The samples were prepared in black 384-well microplates from GREINER (GRE-INER Bio-One GmbH, Bad Haller Strasse 32, 4550 Kremsmünster, Austria) with a filled volume of 70 µl. The enzyme concentration was 100 pM to 100 nM. Incu-

15   bation was undertaken for 30 minutes.

The fluorescence lifetime was measured using an "Ultra Evolution" machine with the fluorescence lifetime (FLT) option from TECAN (TECAN Austria GmbH, Grödig, Salzburg, Austria). The fluorescence laser operated at a wavelength of 440 nm

20   and a repetition rate of 20 MHz. The emission filter was set to a wavelength of 544 nm and a bandwidth of 25 nm and the integration period per well was one second.

25   B)   Results:

Figure 1 shows the measured fluorescence lifetime of the fluorescent marker fluorescein, which is part of the tracer peptide (biomolecule), as a function of the enzyme concentration. Express reference is made here to the fact that no addi-

30   tional auxiliary molecule (such as an antibody or the like) had to be used to ob-tain these measurement results. Measurement was undertaken 30 minutes after the addition of the enzyme PTP1B to the tracer peptide. The fluorescence lifetime reflects the state of peptide phosphorylation, with higher enzyme concentrations working faster and producing more dephosphorylated peptide. In contrast, the

fluorescein bonded to the now dephosphorylated peptide exhibits a shorter life-
time.

5   After a very protracted period and regardless of the enzyme concentration, all
phosphate groups would be removed from the tracer peptide. However, the en-
zyme concentration does determine the exact period required.

Figure 1 also shows that when the enzyme concentration is very low, the state of
phosphorylation is not altered significantly; on the other hand when the highest
10 concentrations are used by adding still more enzymes, no additional effect can be
demonstrated. In this experiment the entire substrate of the sample was con-
verted during the incubation period, so that the proportion of the manifest en-
zyme function is apparent in the center. Figure 1 is based on data for the
dephosphorylation of Panvera tracer P2843 at various phosphatase concentra-
15 tions. The error bars were produced from three independent measurements. The
unbroken line is only to assist appreciation of dependency. The position of this
line moves to the right when the incubation period is shorter and to the left when
the period is longer. The z' value for the data set shown in Figure 1 is 0.62.

20 Figure 2 shows the time-dependent nature of the enzyme reaction, i.e. the en-
zyme kinetics for various enzyme concentrations. Without enzymes the lifetime
signal remains stable, i.e. no dephosphorylation occurs. When there is a moder-
ate concentration one can see the entire process in the selected 6-minute period,
and for very high concentrations the reaction occurs too quickly to be measur-
25 able in this experiment. The measurement points for the various enzyme con-
centrations are shown as diamonds (without enzymes), triangles (1 nM), squares
(3 nM) or spots (30 nM).

In order to verify these results two samples with different fluorescence lifetime
30 signals were measured using fluorescent polarization; no difference in the phos-
phorylation of the tracer peptide could be determined using this known method.

Experiments such as the one just described play an important role in assay de-
velopment or in high throughput screening laboratories. Yet the apparatus previ-

ously available in such laboratories did not permit the measurement of fluorescence lifetimes.

5   As indicated at the outset, an enzyme reaction involving the phosphorylation or dephosphorylation of a peptide is of major biological and pharmacological significance. The present invention provides a novel method for analyzing and representing such important processes. In particular, preferred applications of the method in accordance with the invention include drug discovery screening, that is the discovery, research, optimization or validation and/or the detection of
10   pharmacologically effective substances and/or in conjunction with the corresponding production of pharmaceutical preparations.

   Within the scope of this present invention, attaching phosphate groups to a biomolecule must have a direct impact upon the fluorescent dye. For this to be the
15   case, both groups have to be either in the vicinity of each other or the effect can be translated by means of the conformational transformation of biomolecules, which then modifies the molecular environment of the dye. Both chemical units, the phosphate group as well as the dye, have to be attached to the same biomolecule, for instance covalently. However, the biomolecule can also consist of
20   several subunits, that is (hetero or homo) dimers, trimers, or in general oligomers. In this case the two residues can also be located on different subunits of the same biomolecule.